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(54) Title: CALCIUM PHOSPHATE DELIVERY VEHICLE AND ADJUVANT

(57) Abstract

An improved calcium phosphate delivery vehicle or adjuvant with incorporated adjuvanticity enhancing means and methods of producing same are disclosed. The adjuvant can be fabricated to desired formulations as appropriate and based on the intended purpose. Particle sizes can be adjusted to enhance adjuvant activity. Other supplemental materials may be added as desired and in appropriate proportions to selectively elicit preferred components of the immune system and to enhance the adjuvant's effect on the host response.

Calcium Phosphate Delivery Vehicle and Adjuvant

Field of the Invention

The present invention relates to improved calcium phosphate vaccine delivery vehicles, adjuvants, and methods for producing the same.

Description of the Nearest Art

An ideal vaccine delivery system should be designed to introduce an immunogen to a desired site of action and release it at an appropriate and controllable rate. The carrier should also be non-toxic and should not remain in the host after its use. Both the adjuvant and immunogen should be pharmaceutically stable and the delivery system should be easy to administer. Additionally, the host response to the immunogen should quickly reach maximal levels and result in a protective effect as quickly as possible following vaccine administration.

Traditional vaccines have typically used complex immunogens such as inactivated viruses or bacteria to evoke immunity. Such vaccines were often associated with adverse side effects (e.g. granuloma formation, pyrogenicity, and hypersensitivity). The use of subunit vaccines has reduced the number and severity of unwanted side effects associated with vaccines produced with more complex immunogens. Subunit vaccines are comprised of only one, or a few, proteins or polysaccharides from the target pathogen. Suzue et al in Experientia (77, 1996, pp451-465) teach heat shock proteins (HSPs) as subunit vaccines. Due to their small size, by themselves, subunit vaccines tend to be only weakly immunogenic, often failing to induce a satisfactory level of immunity. Thus to be effective, the use of subunit vaccines require strategies to enhance immunogenicity, such as the use of enhanced adjuvants and specific delivery strategies.

One strategy for improving the immunogenic response to subunit vaccines has been through the use of controlled release vaccine delivery by means of an implantable depot delivery system. Controlled-release technology provides improved

Relyveld in U.S. Patent No. 4,016,252 and in Developments in Biological Standardization, (65, 1985, pp 131-136) describes the production of an injectable calcium phosphate gel, which can adsorb vaccines. The purpose of Relyveld's calcium phosphate gel is to provide highly concentrated and stable vaccines, not to increase adjuvanticity.

Adjuvant particles may be used to effect adjuvanticity of a vaccine. Kreuter et al. reported in Vaccine (6, 1988. pp. 125-129) that a decrease in particle size diameter increased the adjuvant effect of a nanoparticle polymer. Grafe in Arzneim.-Forsch (21, 1971 pp. 903) and Kreuter in Infection and Immunology (19, 1978 pp. 667) reported similar findings using Al_2O_3 adjuvants and γ-ray-polymerized poly(methyl methacrylate) particle adjuvants, respectively. Courvreur et al in U.S. Patent No. 4,329,332 describes synthetic polymeric nanoparticles which contain a biologically active substance that is released to a host at a rate similar to the rate of biodegradation of the polymers. Alemann et. al. in European Journal of Pharmaceutics and Biopharmaceutics, (39, 1993. 173-191) discuss that the continual problem with nanoparticles has been the rapid uptake by the reticuloendothelial system (RES). Since the RES functions to remove small foreign particles from the blood circulation, drugs contained on such particles do not reach their destination site, but rather are found in high concentrations in the liver and spleen. Kreuter in Journal of Controlled Release (16, 1991. pp. 169-176) teaches various methods to avoid RES nanoparticle uptake, such as the use of surfactants, magnetic fields and different administration routes (e.g. subcutaneous, intramuscular and ocular). After subcutaneous and intramuscular injection, nanoparticles treated in these ways stay at the injection site (Kreuter, Journal of Pharmaceutical Sciences, 72, 1983, p1146-1149). Also, drugs and antibiotics were shown to yield reduced toxicity and/or increased efficacy when combined with nanoparticles. It has also been reported by Gurny (Biopharmaceutics of Ocular Drug Delivery, CRC Press, Boca Raton, 1993, p81-90.) that the pre-corneal resident time of pilocarpine was increased after it was incorporated into nanoparticles. Amerongen et al in U.S. Patent No. 5,443,832 also discloses that hydroxyapatite particles of suitable size (0.01 to 0.1 micron) can carry antigens across the epithelium but does not teach adjuvanticity of particle sizes.

"Host Response" - "Host response" refers to the biological reaction generated by a host to a foreign or implanted substance, within a time period, after it is introduced to or recognized by the host. This reaction may be inflammatory, immunological, mitogenic, toxic, or any other reaction in response to the substance. The "host response" may be either adaptive or innate, though both are required for a fully functional response.

"Resorbable" - "Resorbable" is used to describe the elimination of a material from the host. The material can be "resorbed" by enzymatic processes, cellular reaction, dissolution or other biological or physical degradation mechanism. Resorption may take place over a time course from days to years. Resorption as used herein includes remodeling of a substance to bone.

"Antigen" - "Antigen" refers to any substance or active agent that when introduced to a host, elicits, by any means, a specific antibody response to the introduced substance.

"Active Agent" - "Active agent" refers to any substance that has biological activity. Active agents include such substances as antigens, vaccines, tolerogens, immunogens, growth factors, proteins, nucleic acids and others.

"Vaccine" - "Vaccine" refers to any substance that induces an adaptive host response, resulting in immunity or partial immunity to a pathogen. A vaccine may be without limitation a live, attenuated (non-pathogenic) or dead pathogen, an antigen or a subunit vaccine, which is only one or a few proteins or polysaccharides from a pathogen.

"Adjuvanticity Enhancing Means" - "Adjuvanticity Enhancing Means" is a substance, treatment or adjuvant configuration that results in an increased host response to an adjuvant as compared to the host response to the adjuvant in the absence of the adjuvanticity enhancing means. Increased adjuvanticity can often be measured as an enhanced or heightened aspect of some element of the immune or foreign body response (e.g. increase in a specific immune cell type such as, macrophages, CD4, CD8, lymphocytes or specific increases in humoral or cellular pathways).

The enhancing means may be exogenous or endogenous. "Exogenous

shoulders and sharp peaks at 2θ values of 27°-34°. In particular, there are no sharp peaks or shoulders corresponding to Miller's Indices of 210, 112 or 300. Shoulders may be presented at approximately 2θ values of 29° and 33.6°. It is further characterized by FTIR peaks at 563 cm^{-1}, 1034 cm^{-1}, 1638 cm^{-1} and 3432 cm^{-1} (± 2 cm^{-1}). Sharp shoulders are observed at 603 cm^{-1} and 875 cm^{-1}, with a doublet having maxima at 1442 cm^{-1} and 1457 cm^{-1}.

"Nanoparticle" - The term "nanoparticle" used herein describes the physical diameter of an adjuvant particle. A nanoparticle ranges from 1.0 nm to 1000 nm or 1.0 µm. As discussed herein, adjuvant size refers to the average nanoparticle size, unless expressed otherwise, as determined by scanning electron microscopy.

Detailed Description of the Invention

The present invention provides calcium-containing adjuvants and vaccine delivery vehicles which, when present alone or in combination with one or more active agents such as antigens or vaccines, elicit a host response or augment a host response towards the antigen or vaccine. The inventive adjuvants or delivery vehicles may provide continuous, delayed, sequential and/or intermittent depot delivery of an antigen or other active agent to a host. In other cases, the material may deliver a quick one-time dose of an active agent to a host.

Characteristics of the Calcium-Containing Adjuvant

The adjuvant is a calcium-containing material. Any calcium compound can be used, although calcium phosphates and calcium sulfates are preferred. Amorphous and poorly crystalline apatitic calcium phosphates are particularly preferred. In some instances, additional materials in conjunction with the calcium based adjuvants may be present to augment adjuvanticity. Generally, the calcium compound is formed into an injectable gel or solid nanoparticles. The preferred adjuvant or vehicle can be designed to absorb, bind, entrap or otherwise contain or present an antigen, subunit vaccine or other active agent. Useful calcium adjuvants include, but are not limited to, calcium sulfates and calcium phosphates such as amorphous calcium phosphates (ACP), poorly crystalline apatitic calcium phosphates (PCA), dicalcium phosphate dihydrate (DCPD), tricalcium phosphates (TCP), tetracalcium phosphate (TTCP), monetite, monocalcium phosphate

the needs of a specific application. In some instances, the calcium-based adjuvant will be non-resorbable and will remain in the body indefinitely. Ceramic adjuvants, such as sintered calcium phosphate (e.g. highly crystalline hydroxyapatite), remain in the body after completion of vaccine delivery. In preferred embodiments, the adjuvant is resorbable. Resorbable adjuvants biodegrade over time, ultimately leaving little or no residual material in the body. Adjuvants may be either strongly resorbable or weakly resorbable. In preferred embodiments of the invention, a strongly resorbing adjuvant is characterized as follows: at least one gram (preferably 1-5 g) is implanted in a subcutaneous or intramuscular site, at least 80% of the adjuvant is resorbed within one year. In more preferred embodiments, one gram of adjuvant will be resorbed within nine months, six months, three months, and ideally one month or less. Weakly resorbable means that less than 80% of one gram of starting adjuvant is resorbed after one year. Resorption, as used herein, encompasses solubility based dissolution processes, as well as active cellular or enzyme based processes. Preferred calcium-based adjuvants are resorbed through active cellular or enzymatic processes. By controlling the rate of active degradation of the adjuvant, the inventive adjuvants can be tailored to have linear resorption rates.

Resorbability of the inventive calcium phosphate vehicles can be varied through the adjustment of one or more physical parameters including vehicle size, vehicle particle size, porosity, density, and/or crystallinity. Two or more of these parameters will generally be adjusted in concert to fine-tune the final resorption rate. Additionally, certain molecular factors may be incorporated into the vehicle that can be used to affect its resorption rate by influencing the cellular or enzymatic processes that ordinarily mediate vehicle resorption in the body. These incorporated factors are often biologically active molecules or collections thereof, which affect bone metabolic processes, such as the activity of osteoclasts and/or osteoblasts. In other instances the incorporated factors attract or otherwise affect the activity of one or more of macrophages, monocytes, or foreign body giant cells. Such useful factors include: growth factors, enzyme inhibitors, extracellular matrix components, cytokines and the like.

Ultimately, resorption rates will be established empirically by using

adjustment to regulate resorption rate may not be feasible and it may be more advantageous to further control, or fine-tune, resorption rates by altering other physical parameters, such as adjuvant density or crystallinity or through the addition of proteins or growth factors which affect resorption rates.

Density. Vehicle density also has a significant effect on resorption rates. Density is most easily controlled by compression of the adjuvant following fabrication. Compressive forces of 8 MPa to 50 MPa may be applied through the use of molds and presses. In addition to compression, a variety of other methods, which are useful to adjust vehicle density, are known to the art and may be used. Calcium phosphate density for the inventive adjuvants and vehicles is best determined using pycnometry, such as Helium pycnometry (HP density). Calcium phosphates prepared with HP densities of approximately 3.0 gm/cm^3 will be useful for the production of slow resorbing vehicles. More rapid resorbing vehicles may be prepared from calcium phosphates with HP densities generally in the range of 2.5 - 2.8 gm/cm^3, preferably 2.5 gm/cm^3. HP densities with values less than 2.5 gm/ cm^3 are often preferred for vehicles that are intended to resorb particularly rapidly.

Crystallinity. Generally, careful control of the adjuvant's degree of crystallinity and crystal size may be used to affect the overall vehicle resorption rate. For apatitic calcium phosphates with calcium to phosphorous ratios of 1.3-1.75, poorly crystalline forms are believed to resorb more quickly than highly crystalline forms. Highly crystalline stoichimetric hydroxyapatite (e.g. NIST® catalog # 2910) is an example of a weakly resorbable vehicle. For other calcium phosphates, for a given calcium to phosphorous ratio, more amorphous forms will generally be more soluble than more crystalline forms. Increased resorption rates may be achieved through the production of apatitic calcium phosphates containing lattice defects, such as ionic vacancies or substitutions. Preferred embodiments include carbonated or otherwise calcium deficient apatites, all of which tend to have increased in vivo resorption rates. Further guidance for the production of similar such apatitic calcium phosphates can be found in <u>Structure and Chemistry of the Apatites and Other Calcium Orthophosphates</u>, (Elsevier, Amsterdam, 1994, by J.C. Elliott), and the references contained therein, all incorporated herein by reference.

μm -300 μm in diameter to the calcium phosphate of examples 16 and 17 at proportions of 20% weight percent. These porogens may be leached from the adjuvant, post fabrication.

Incorporation of resorption factors.

Incorporation of factors, which attract or inhibit osteogenic cells and/or macrophages, can have a significant effect on adjuvant resorption rate. Thus, incorporation of bone morphogenetic protein into the inventive adjuvants will lead to more rapid resorption of the vehicle, particularly in soft tissue implant sites. Additionally, factors that attract osteoclasts (e.g. interleukin-1, lymphotoxin, calcitonin) may be used to promote degradation of the vehicle. Osteoclast or macrophage activity inhibitors (e.g. neutral phosphate, glucocorticoids, plicamycin, gallium nitrate) may be used to prolong the resorption process. Extracellular matrix components, such as laminen, RDG peptides, collagen, fibronectin may also be included with the adjuvants. Further guidance regarding specific factors useful in the regulation of calcium phosphate resorption rates can be found in PCT/US97/18528, incorporated by reference herein. Generally, these factors will be incorporated into the inventive adjuvants as a concentration of less than 20% wt/wt preferably less than 10% and in most embodiments, less than 5%.

In many instances, adjuvant resorbability is preferred; however, it is not always required or desired. In some embodiments the inventive adjuvanticity enhancement can be obtained with a calcium adjuvant that is either weakly resorbable or non-resorbable. A non-resorbable adjuvant may be used when prolonged antigen delivery will occur over a matter of several years. A non-resorbable adjuvant may also be desirable in cases when the adjuvant is used additionally as a support matrix for tissue repair or growth, as a treatment for a disease, or for vaccination purposes. Non-resorbable calcium phosphate adjuvants can remain in the body without detrimental effects to the host due to their excellent biocompatibility. Alternatively, non-resorbable adjuvants may be surgically removed following the desired delivery period. Suitable non-resorbable or weakly resorbable calcium phosphate adjuvants include those prepared from sintered hydroxyapatite.

preferred embodiments, the particle size ranges from about 0.1nm to about 900 nm, more preferably 1.0 nm to about 500nm, and most preferred from about 100 nm to about 250 nm. Optimally, the particle size ranges from about 20 nm to about 80 nm. In some cases ranges of 5 to 50 microns have been useful to enhance adjuvant activity. Larger particles (50 μm or greater) typically employ other adjuvanticity enhancers, such as cytokines, other adjuvants or physical parameters (e.g. porosity), are included to enhance the host reaction.

Depot delivery systems with varying particle sizes may be used to avoid rapid RES uptake and thereby providing a more efficient delivery method. For those embodiments where a specific immunogen is adsorbed or covalently linked to the adjuvant, the release profile of the immunogen from the adjuvant exhibits sensitive dependency on particle size. Small particles (e.g. nanoparticles) can release the antigens at a higher rate than larger particles because of their larger surface-to-volume ratio. The larger particles (e.g. microparticles) can not be engulfed by phagocytic accessory cells until they are degraded into smaller particles; therefore, the larger particles serve as a depot of continuous antigenic stimuli. The rate of antigen release from these larger particles is at least indirectly dependent on the resorption rate of the particles themselves. In one embodiment, a combination of small and large particles may create a pulsatile pattern of antigenic release, thereby mimicking the antigen concentration profiles typically seen during immunization scenarios that utilize an initial primary shot and followed by a number of booster shots.

Particle Shape. The shape of the individual particles and of the adjuvant itself may endogenously affect the adjuvanticity. Generally, ACP adjuvants contain particles that appear spherical and/or sometimes fused. Spherical ACP particles are prepared according to Example 1. PCA calcium phosphate particles are usually needle-like in appearance. Adjuvants containing needle-like particles will generally produce a greater response when compared to adjuvants containing only platelet-like or spherical particles. In some preferred embodiments, the adjuvant will comprise needle-like particles prepared according to Example 12. In other cases, both spheres and needles, as well as plate-like particles may be used. Adjuvant particles prepared according to Example 13 represent the combination of different particle shapes.

a greater immune response for complete protection or attack. Additionally, in other technologies, such as bone growth, surface roughness provides an improved adhesive structure, or scaffold, for the attachment of bone forming cells (Suzuki, 1997). This concept is adapted to the surface of an adjuvant to attract and secure cells associated with the immune response. The degree of particle roughness can be determined by scanning electron microscopy as well as other sophisticated surface measurement instrumentation. In preferred embodiments, a loaded adjuvant with a rough surface will provide an adhesive structure for the attachment of desired immune cells (e.g. antibodies).

pH. In most cases, the adjuvant's pH is neutral and will not by itself elicit a host response. Adjuvant pH will be varied depending on the application of the adjuvant and the antigen in use (the stability of certain antigens is affected by pH). Generally, adjuvant pH is about 4.0 to about 10.0; more preferably, pH values should be about 6.0 to about 9.0. In some embodiments, adjuvant pH may be more acidic (e.g. 0-3) or basic pH (e.g. 11-14), in order to elicit a pyrogenic or cytotoxic host responses.

In preferred embodiments, the endogenous pH of the calcium-based adjuvant is adjusted with raw materials or precursors during manufacturing. The preparation and ratios of starting materials can be varied to control the pH of the adjuvant. In other cases, the pH of the precipitate or gel can be adjusted. In preferred embodiments, calcium salts will be selected with pK_as at or near the desired pH of the adjuvant. In other embodiments, the pH is lowered by adding an acid or acidic buffer (e.g. phosphoric acid, hydrochloric acid, monobasic sodium phosphate acetic acid) or the pH is raised by adding a base or alkaline buffer (e.g. potassium hydroxide, calcium hydroxide, sodium hydroxide), or through the use of buffering agents with pK_as in desirable ranges as is known in the art. In yet other embodiments, the pH of an adjuvant in paste or slurry form can be controlled by introducing specific pH buffered solutions as hydrating agents. Since extremes of pH may weaken the electrostatic interactions of the antigen-to-antibody binding, in preferred embodiments, the pH of the adjuvant will correlate with the pH of the antigen-to-antibody complex. However, in rare embodiments, the adjuvant

1992, p2357-2362).

Example 24 describes the use of exogenously added cytokines/adjuvant combinations used to combat tumors. A persistently high concentration of cytokines (e.g. IL-2, IL-4, IL-6, IFN-γ, TNF-α, GM-CSF) in the vicinity of tumor cells is known to stimulate antitumor activity by amplifying the immune response to the tumor by both antigen-specific and nonspecific T cells. By coordinating the immune response with the appropriate inventive adjuvant, cytokine gene-based cancer vaccines induce long-term systemic antitumor activity, in addition to generally eradicate injected tumor cells. The exogenous incorporation of cytokines, such as gamma interferon and IL-12, into the inventive calcium phosphate adjuvants may boost both the humoral and cellular responses to an incorporated antigen. IL-1 increases antibody production by directly stimulating B lymphocytes and is known to potentiate T-cell proliferation by increasing the production of IL-2. IL-6 has the ability to stimulate immunoglobulin production. IFN-γ activates helper T cells. IL-12 has been demonstrated to increase cell-mediated immunity for the control of virus infections. Further guidance for the use of cytokines in adjuvants can be found in Vaccine Design, (Powell, Plenum Press, New York, 1995) and ImmunoBiology, Appendix II, (Current Biology Ltd./Garland Publishing, New York, 1996) incorporated in their entirety by reference. Controlled-release calcium phosphate adjuvants are particularly useful for the delivery of cytokines to regulate or inhibit tumor growth. Antigen encapsulated in large microspheres (20-40 μm) can be used for slow release of the antigen, while antigens encapsulated in submicron particles (< 1μm) can be used to induce rapid macrophage uptake and processing. The use of an adjuvant which specifically induces cytokine release, thereby results in a response augmentation is also considered to be within the scope of this invention.

In some embodiments, the calcium-based adjuvant may be prepared in combination with an exogenous adjuvanticity enhancing means. More specifically, an adjuvanticity enhancer may be another known adjuvant, such as aluminum hydroxide, aluminum phosphate, muramyl dipeptide, biodegradable polymeric microspheres, liposomes and others. For example, Complete Freund's adjuvant (CFA) activates Th1 cells resulting in delayed-typed hypersensitivity, while alum activates Th2 cells,

producing microspheres. Methods of encapsulation, using polymers and liposomes, are well known to those skilled in the art. The size of the microspheres is controlled during manufacturing. The coordinated use of smaller microspheres (<10 μm) and larger microspheres (> 10 μm) will create the pulsatile kinetics of antigen release typically seen with primary and secondary immunizations and boosters, respectively. The antigen may be combined (incorporated or adsorbed) with the liposome or polymer, the calcium phosphate adjuvant or both. The calcium phosphate/liposome adjuvant may also entrap any substance that will improve the vaccine, such as desired cytokines, other adjuvants, and composite materials.

Composites.

In some instances, it may be desirable to alter specific physical properties of the calcium-based adjuvant to an extent not possible with the pure adjuvant material alone. In such instances, the adjuvant may be used in a composite form through the addition of one or more supplemental materials. A supplementary material is a substance that is added to the inventive adjuvant and alters a physical property or characteristic of the adjuvant such as tensile strength, elasticity or flexion. In some instances the supplemental material may also serve as an enhancing means. The supplemental material may be supplied in any suitable form (e.g. particulate, fiber). In preferred embodiments, the supplementary material is added to the inventive calcium phosphate adjuvant at volume fractions of 1-50% and more preferably, 1-20% either during synthesis or after manufacturing. In embodiments that encompass resorbable calcium phosphate adjuvants, it is preferred that the supplemental material is bioresorbable as well; however, the resorption rates may vary.

Composite adjuvant systems may be designed with delivery kinetics similar to those observed with traditional primary/booster immunization strategies. In such embodiments, the antigen is released at calculated intervals and/or rates, to induce proper antibody response levels associated with optimal immunization. Such composite adjuvants are useful since they comprise the desirable characteristics of both more resorbable and less resorbable adjuvant. As an added option, the relative ratios may be varied to produce vehicles of differing resorption profiles. In some

because RES uptake of the inventive adjuvant is avoided through the use of surface treatments, coatings, surfactants and/or magnetic fields. Non-ionic surfactants such as poloxamers (e.g. Poloxamer 188 and Poloxamer 338) have been used to coat the surfaces of nanoparticles to delay RES uptake. In a preferred embodiment, the calcium phosphate adjuvant/immunogen-nanoparticles are suspended in saline or water containing 1% of the chosen poloxamer, which adsorbs strongly to the surface of the nanoparticles. The coated particles are particularly useful for intravenous administration. Coated particles are more likely to reach other target sites (e.g., tumor tissue) than uncoated particles, especially when injected intravenously. Other suitable coatings and surfactants known in the art may also be useful for directing antigen-loaded particles to desired sites. Further guidance for using surfactants can be found in Illum (FEBS Letters, 167, 1984. p79-82) and Leu (Journal of Pharmaceutical Science, 73, 1984. p1433-1437).

In some cases, it may be desirable to combine magnetic materials with the inventive calcium phosphate adjuvants to guide the controlled delivery of the active agent to the target site. A magnetic field is placed around the target to improve efficacy. The magnetic adjuvant particles are attracted to the magnetic field and tend to concentrate around the target, thereby delivering the active agent focally to the appropriate site. In one example of this embodiment, the calcium phosphate adjuvant/antigen-nanoparticles are combined in distilled water or saline with an appropriate amount of magnetite. The coated nanoparticles are administered intravenously. In most cases, tumor sites are specifically targeted although any site can be chosen. Further guidance for using magnetic materials can be found in Widder (European Journal of Cancer and Clinical Oncology, 19, 1983, p135-139 and p141-147) incorporated herein by reference.

In other embodiments, composites of calcium salts and any additional materials are prepared as a paste by the addition of a fluid to a mixture of the calcium adjuvant and the supplemental materials. The paste is then hardened by drying or in conjunction with a hardening reaction.

Manufacturing the Adjuvant.

The calcium adjuvants of the present invention can be manufactured to

maturation times. Higher temperatures usually yield more crystalline particles.

Alternatively, the gel can be lyopholized to form a dry powder. The powder's (either lyophilized or non-lyophilized) particle size can be controlled by sieving, milling and treating with different temperatures. Sieving the powder allows the particles to be separated according to size, usually from smallest to largest. In preferred embodiments, the powder is milled. In most cases, a longer milling time results in a smaller particle size. In other embodiments, the powder is heated to different temperatures. Higher temperatures (e.g. 400°C-600°C) can be employed to increase the crystallinity of ACP powders. However, for temperatures up to about 450°C, the amorphous character of an ACP is preserved, but the specific surface area decreases, which marks a decrease in particle size. In other embodiments, a hardened form of the calcium-based adjuvant may be broken down into particles by milling, pulverizing and other methods. Fine powders of the nanometer or less size range (e.g. ACP), can be compressed in a mold (e.g. compressive strength: 500 psi), then milled and sieved to achieve desired particle sizes. Other methods known in the art used to control particle size are considered to be within the scope of the invention.

Loading the Calcium Phosphate Adjuvant with the Active Agent.

The adjuvant can be combined with one or more active agents, immunogens as well as adjuvanticity enhancers. The active agent with additional moieties may be combined with the calcium-based adjuvant through dissolution, adsorption, co-precipitation, centrifugation in a hydration medium, encapsulation, diffusion based processes, or any method known in the art. In preferred embodiments, the active agent is adsorbed during manufacturing onto calcium adjuvants in the presence of a buffering system compatible with the specific active agent. In preferred embodiments adsorption will occur under condition of relatively low ionic strength (e.g. 0.001 M-0.2 M NaCl) in the presence of minimal buffer concentration (e.g. 0.001 M TRIS pH 7.0) and generally with the presence of little or no phosphate ions (e.g. < 0.1M). Further guidance, incorporated herein by reference, for adsorbing active agents can be found in Relyveld in U.S. Patent Nos. 3,925,545 and 4,016,252 and Relyveld in Developments in Biological Standardization (65, 1985, pp. 131-136). Towey et al., in U.S. Patent No. 2,967,802, describes the method

In yet another embodiment, the antigen and other moieties if present, will be encased or encapsulated by the adjuvant. In such embodiments, the adjuvant will act as a capsule for containing the antigen. Since the adjuvant is resorbable, the antigen will be introduced to the body when the adjuvant is depleted, providing the host with a dose of antigen. In some embodiments, the finished adjuvant vehicle/device will contain the active agent in a layered form. Such layers can be designed so the active agent is released sequentially from the layers allowing the administration of multiple doses. Generally, devices prepared in this format will rely on solubility characteristics of the layers for proper delivery characteristics. In still other embodiments where the adjuvant is both permeable and resorbable, the encapsulated immunogen will diffuse out of the adjuvant/vehicle in a controlled delivery fashion until the encapsulation is breached by resorptive processes. At such time any remaining immunogen is released in a final delivery pulse. Specific embodiments for the production of vehicles employing layered architecture or encasing active agents using solid free-form technology as described by Cima in U.S. Patent No. 5,490,962 and 5,518,680, incorporated herein by reference. Solid free-form technology can modify the adjuvant properties (e.g. release rates, adjuvanticity properties) by changing the three dimensional shape of the adjuvant. This technology is useful for controlled release of a bioactive agent and implantation and growth of cells. In one solid free-form embodiment a resorbable calcium phosphate powder such as is described in issued U.S. patent 5,676,976 by Lee et. al., incorporated herein by reference, is used as a matrix to encapsulate less resorbable calcium phosphate powders; which have been preloaded with an immunogen. The immunogen is also present in the matrix. The presence of the slower resorbing calcium phosphates insures the long term delivery kinetics of the immunogen.

In some embodiments, it may be desirable to modify the surface of the calcium adjuvant in order to improve the interface between the adjuvant and the active agent. The adjuvant may be subjected to surface treatments, such as plasma etching or sputter coating to alter the interfaces between the two phases as is known in the art. Surface treatments may be used to increase or enhance the affinity of the adjuvant for active agents such as proteins. Plasma etching may provide an altered or rough

vaccines, second adjuvants, bacteria or viruses, or fragments thereof, nucleic acids, proteins, heat shock proteins (HSPs), haptens, tolerogens, allergens, immunogens, antibiotics, and other bioactive moieties or components of biosynthetic pathways. A more complete listing of compounds suitable for delivery to a host can be found in Vaccine Design, The Subunit and Adjuvant Approach (Powell et al. (eds), Plenum Press, New York, 1995), which is incorporated herein by reference.

In some embodiments the inventive adjuvants will be used to stimulate an immune response in the absence of an active agent. For example, local implantation of an active agent-free adjuvant near a tumor may suffice to recruit a local reaction sufficient to reject the tumor. In these instances it may be advantageous to employ one or more adjuvanticity enhancers or enhancing strategies , such as the incorporation of a specific cytokine and the use of a rough surfaced vehicle. In other cases, the adjuvant is introduced to the host either before or after the antigen, but the adjuvant and the antigen are not combined as a single entity.

Dose Issues.

The correct dose of immunogen to be delivered by the inventive adjuvant must be determined for each application. An absolute dose is the total amount of an active agent loaded onto the adjuvant, whereas a chronic dose is the amount of active agent released and delivered to the recipient per unit of time. The relative importance of these two aspects of dose depends on the purpose of administration and properties of the active agent. Generally, larger doses of vaccines may be required to establish immunological memory. Dosage is preferably adjusted in conjunction with the rate of adjuvant resorption: Faster resorbing adjuvants will tend to deliver dosages more quickly than a slower resorbing adjuvant because it is introduced into the host more rapidly and at an increased concentration. In preferred embodiments, the adjuvant's resorption rate will mimic a traditional initial immunization followed by subsequent or booster immunizations. The length of time between immunizations, called a rest period, is usually necessary for the induction of antigen-specific lymphocytes, particularly memory B lymphocytes, and an effective vaccination. Generally, a longer rest period (e.g. several weeks or months) will produce the maximum antibody response.

Uses-Depot delivery and controlled release.

The inventive adjuvant offers the advantage of either site specific delivery or systemic delivery. Once introduced to a host, the adjuvant/device will deliver the active agent. The inventive adjuvant delivers the antigen with the desired kinetics. In preferred embodiments, the adjuvant will provide a depot delivery of the antigen to the host. Additionally, the adjuvant will provide site-specific delivery of the antigen. Target site specific delivery is known to improve therapeutic efficacy of an active agent and reduce undesired side effects. Specific targets include tumor tissue, prior or subsequent sites of active agent injection and/or any site where rejection is desired or in the case of vaccination to the specific antigen preferred sites are those which lead to optimal immunization(e.g. spleen, liver, kidney, lymph nodes, etc.).

The invention is further exemplified with reference to the following examples, which are presented for the purpose of illustration only and are not to be considered as limiting of the invention.

Example 1. This example describes the preparation of an amorphous calcium phosphate (ACP)adjuvant.

Solution A was prepared at room temperature by the rapid dissolution of 55g $Na_2HPO_4{\cdot}7H_2O$; 50g NaOH; 30g $NaHCO_3$ in 1.3 liters of distilled water. Solution B was prepared at room temperature by rapid dissolution of 43g $Ca(NO_3)_2{\cdot}4H_2O$ in 0.5 liters of distilled water.

Carbonated amorphous calcium phosphate was then prepared at room temperature by the rapid addition of solution B to rapidly stirring solution A. The precipitate of gel-like amorphous calcium phosphate thus formed was aged or allowed to stand at room temperature for approximately 30 seconds. After aging, the precipitate was filtered using filter paper (0.05 m^2) with medium filter speed and a vacuum pressure of about 10^{-2} torr. The precipitate formed a thin cake and was washed with approximately 4 liters of distilled water by adding water into the filtrating funnel. The gel pH was measured using a pH probe and determined to be pH 13.5

Example 2. This example describes the preparation of an amorphous calcium phosphate adjuvant.

immersed into a liquid nitrogen in a 2.5 L container. Following freezing, the material was transferred into a vacuum chamber for 24 hours (10^{-1} - 10^{-2} torr), until a fine and dry powder was obtained.

Example 6. This example describes the preparation of an amorphous calcium phosphate (ACP) adjuvant.

The ACP adjuvant was prepared according to example 5 but with the following modification. The precipitate was aged at room temperature for 30 minutes.

Example 7. This example describes the preparation of an amorphous calcium phosphate adjuvant.

The ACP adjuvant was prepared according to example 1 but with the following modifications. Solution A was prepared at room temperature by the rapid dissolution of 55 g $Na_2HPO_4 \cdot 7H_2O$; 20 g NaOH; 50g $NaHCO_3$; 2 g $Na_2P_2O_7$ in 1.3 liters of distilled water. Solution B was prepared at room temperature by rapid dissolution of 100 g $Ca(NO_3)_2 \cdot 4H_2O$; 1 g $MgCl_2 \cdot 6H_2O$ in 0.7 liters of distilled water. The gel pH was measured using a pH probe and determined to be pH 9.0.

Example 8. This example describes the preparation of an amorphous calcium phosphate (ACP) adjuvant.

The ACP adjuvant was prepared according to example 7 but with the following modifications. The precipitate was aged at room temperature for 30 minutes. The washed material was then collected using a spatula and immersed into a liquid nitrogen in a 2.5 L container. Following freezing, the container was transferred into a vacuum chamber for 24 hours (10^{-1} - 10^{-2} torr), until a fine and dry powder was obtained.

Example 9. This example describes the preparation of an amorphous calcium phosphate (ACP) adjuvant.

The ACP adjuvant was prepared according to example 7 but with the modification that the precipitate was aged at room temperature for 2 hours.

Example 10. This example illustrates the typical formation of a calcium phosphate apatite adjuvant.

A solution of 218 g of disodium $Na_2HPO_4 \cdot 12H_2O$) in 1.2 liters of distilled

Following freezing, the precipitate was transferred into a vacuum chamber for 24 hours (10^{-1} - 10^{-2} torr), until a fine and dry powder was obtained.

Example 15. This example illustrates the typical formation of a calcium phosphate apatite adjuvant.

The calcium phosphate apatite adjuvant was prepared according to example 10 but with the modifications that the pH of the precipitating solution was adjusted to pH 7.1 with the addition of sodium hydroxide prior to filtration and the precipitate was aged at room temperature for 48 hours.

Example 16. This example illustrates the preparation of an apatitic calcium phosphate adjuvant.

Dicalcium phosphate dihydrate (DCPD) was prepared at room temperature by the rapid addition of solution B (17.1g $Ca(NO_3)_2 \cdot 4H_2O$; 0.250 liters distilled water; pH 5.5-6) to a stirred solution A (10 g $H_9N_2O_4P$; 0.5 liters distilled water; pH 7.8). Immediately thereafter, the sample was filtered using filter paper (0.05 sq. m) with medium filter speed and a vacuum pressure of about 10^{-2} torr. The material formed a thin cake which was washed with about 2 liters of distilled water and then dried at room temperature for 24-72 hours.

Reactive amorphous calcium phosphate was prepared according to example 1. The washed material was then collected using a spatula and immersed into a liquid nitrogen in a 2.5 L container. Following freezing, the material was transferred into a vacuum chamber for 24 hours (10^{-1} - 10^{-2} torr), until a fine and dry powder was obtained. The material was then heated for 80 minutes at 455° (±3°C)

The reactive amorphous calcium phosphate material was physically dry-mixed with $CaHPO_4 \cdot 2H_2O$ at 50:50 weight percent using a mortar and pestle for 3-5 minutes. Water (1 ml/g of mixed material) was then added to the powder mixture to yield a hydrated precursor of paste-like consistency. The amount of H_2O added varied, depending on whether a thick or thin paste was desired. The paste material was then placed in a moist tissue environment where upon reaching body temperature (37°C), it hardened into a solid mass. The hardening process could be delayed for several hours by placing it into a refrigerating temperature of 4°C.

Example 17. This example describes the preparation of the PCA calcium

right injection sites of terminal mice were directly embedded in OCT medium, frozen and stained with CD3e, CD4 and CD8a. Left injection site of terminal mice were immediately fixed with 10% neutral buffered formalin for 6 to 24 hours followed by embedding in paraffin wax and staining with hematoxylin and eosin and CD45R/B220 and Mac-3. Tissue was cut to 6 um sections with a cryostat for frozen tissues or rotary microtome for paraffin-embedded tissues, and stained for 60 minutes with Pharmingen monoclonal rat or hamster anti-mouse primary antibodies against Mac-3 or several CD antigens (CD3e, CD4, CD8a and CD45R/B220).

A labeled streptavidin-biotin complex/HRP detection system (Dako No. K377) with an AEC chromogen was used to visualize the antigens. Sections were counterstained with Gill's III hematoxylin. Anti-hamster IgG (for CD3e), goat anti-rat Ig (for CD8a, CD45R/B220 and CD4) and mouse anti-rat IgG1/IgG2a (for Mac-3) and hamster IgG isotype standard (for CD3e), were appropriately diluted and substituted as the primary antibody on negative reagent control tissue sections in order to verify the specificity of the reaction.

The immunohistochemical staining was evaluated with a semi-quantitative technique using N (negative) and P (positive) identification protocols. A cell was considered positive when there was a cytoplasmic red fuschia staining and a cellular morphology corresponding to the cell stained with the various CDs or Mac-3 in the positive control tissues.

examples 1 and 7. Mac-3 positive cells were observed primarily adjacent to the test adjuvant and often infiltrated the peripheral region of the adjuvant. They sometimes contained intracytoplasmic material consistent with the adjuvant, possibly suggesting that Mac-3 positive cells (macrophages) were primarily involved in the resorption of the various adjuvants.

The B lymphocytes, detected with CD45R/B220 antibody, were the second most numerous type of cells seen at the injection sites. The incidence of the groups with positive B lymphocytes as well as the amount of CD45R/B220 positive cells in these groups (usually graded as slight to moderate) increased at days 7 and 14 compared to the 72 hours euthanasia time period. The lowest amount of B cells was noted at the injection site of the mice receiving adjuvants prepared according to examples 6 and 8. More specifically, the B cell reaction can be grouped as delayed, strong or transient. Example one produced a strong B cell reaction at 7 days. The adjuvants of examples 3 and 5 produced a delayed reaction. Transient B cell reaction was observed in examples 7, 12 and 15. The presence of a relatively high number of B lymphocytes at the injection sites may suggest that the mediators of the humoral immune response may play an important role in the test article-related inflammatory process.

A time-related increase incidence of the groups with positive T lymphocytes (CD3e, CD4 and CD8a) was observed. After each euthanasia period, CD4 was the most common type of positive T cell observed in the various adjuvants. At 7and 14 days post treatment, CD3e was the second most commonly observed T cell.

These results are reported in the following Table 2:

	72 hours	7 days	14 days
CD3e	2	6	9
CD4	4	7	11
CD8a	3	5	7
Total # of treated groups	12	2	12

capsule formation appeared to be present at the injection site of the mice receiving the calcium phosphate adjuvants of examples 3, 5, 7, 11, and 15 in all recovery periods compared to the mice receiving other formulations. Mononuclear inflammatory cells were always seen at the injection sites and were graded as slight to moderate. Multinucleated giant cells were primarily seen in mice euthanized at 7 and 14 days post treatment and were graded as slight to moderate. In the majority of the calcium phosphate adjuvanted groups, there was usually a time-related increased severity in the infiltration of mononuclear inflammatory cells and multinucleated giant cells, and in the fibrous capsule formation. Neutrophils and eosinophils were primarily seen in mice euthanized after 72 hours of recovery periods and were graded as slight to mild. There was a time-related decreased severity in the infiltration of eosinophils and neutrophils. The amount of adjuvant at the injection site was graded as mild to severe (or marked). There was a slight to mild decrease of adjuvant in the various treated mice euthanized on days 7 and 14 compared to those euthanized at 72 hours.

Example 19. This example shows how *Bordetella pertussis* is loaded into the adjuvant of the present invention to prepare a vaccine for whooping cough.

All solutions were prepared sterilized. The calcium adjuvant is prepared according to examples 1-16, with the following modifications. The *Bordetella pertussis* (commercially available from Pasteur Vaccins) bacilli; killed and centrifuged, are homogenized in a 0.07 M dibasic sodium phosphate sterile solution so as to obtain 4×10^{10} bacilli per ml. The bacterial suspension of germs thus obtained is mixed with Solution A prior to mixing with Solution B. *B. pertussis* becomes absorbed the calcium phosphate precipitate of the present invention.

Example 20. This example describes the adsorption of an active agent onto the surface of the adjuvant.

The calcium phosphate adjuvant is prepared according to examples 16 or 17. The precipitate is hardened at 37°C. The *Bordetella pertussis* (commercially available from Pasteur Vaccins) bacilli; killed and centrifuged, are homogenized in a 0.07 M dibasic sodium phosphate sterile solution so as to obtain 4×10^{10} bacilli per ml. The adjuvant (hardened calcium phosphate precipitate) is placed (e.g. dipped) into the suspension containing the *B. pertussis* and therefore adsorbed onto the surface of the

Vaccins) is attached to the adjuvant according to example 19. An adjuvant dose of 0.5 mL is injected subcutaneously into a mouse. The mouse is subjected to whole polio virus 3 months later. The mouse is evaluated for survival.

Example 29. This example illustrates the use of the inventive adjuvant used to deliver a live attenuated virus.

The calcium phosphate adjuvant is prepared according to examples 16-17. Average particle size of 5μm is selectively sieved. The vaccine is a cell-free preparation of OKA strain varicella(chickenpox)-zoster virus (VZV), available from Pasteur Vaccins. VZV is included in the hydration media at a concentration of 5.0 mg/ml. 2.0 ml paste vaccine is subcutaneously administered by injection. After 4 weeks, blood samples are taken and serum is analyzed for VZV antibodies using enzyme immunoassay (EIA), latex agglutination (LA), indirect fluorescent antibody, and fluorescent antibody membrane antigen assays.

Example 30. This example demonstrates the delivery of more than one antigen using the inventive calcium adjuvant.

The calcium adjuvant was prepared according to example 1and example 17. Commercially prepared hepatitis B vaccine (Hevac B) and diptheria-tetanus-pertussis (DTP)-polio vaccine are available from Pasteur Vaccins. The vaccines are inactivated with formaldehyde and for the adjuvant of example 1 are combined with the adjuvant during the mixing of Solutions A and B. For the adjuvant of example 17 the vaccine is included in the hydration media at a concentration of 5.0 mg/ml. The paste is subsequently hardened at 37°C in a humid environment, then pulverized to a particle size of 250 nm. The prepared hydrated adjuvant is injected subcutaneously. A second injection is given 6 months later and a third injection again in 6 months. Blood samples are taken the day of the first injection and each subsequent injection. HB virus seric markers (HbsAg, anti-HBs, anti-HBc) are tested for with commercial radioimmunoassays (Abbott Laboratories). Anti-toxin titers to tetanus and diphtheria are determined by the passive hemagglutination techniques using highly purified toxins that are coupled to turkey erythrocytes by glutaraldehyde. For determination of pertussis agglutinins, measurements are made using the agglutination test performed in microtiter plates.

schedule. Blood samples are collected 24 hours after each immunization and the sera is isolated. At each time point, anti-hepatitis A antibody titers are measured using ELISA.

Example 34. This example illustrates the use of a combination of weakly and strongly resorbable calcium phosphates in one adjuvant.

Strongly resorbable calcium phosphates are prepared according to examples 16. Weakly resorbable calcium phosphate material is prepared according to example 1,2, or 3 in Ison 5,683,496 or example 4 or 8 in Chow 5,522,893. Both materials are ground separately in a SPEX 8510 laboratory mill with a SPEX 8505 alumna ceramic grinding chamber for 2 minutes. All material is sieved to collect 100 nm to 250 nm sized particles. A 1:5 mass or volume ratio of weakly and strongly resorbable particles is prepared as an injectable slurry with an appropriate amount of buffered solution containing 0.5 mg/ml of keyhole-limpet hemocyanin. The paste is stored for one hour to allow adsorption. 0.5 ml of loaded adjuvant paste is injected subcutaneously into a mouse. Blood samples are collected and sera are isolated at time-points 7 days, 14 days, and 3 months. Anti-keyhole limpet hemocyanin antibody titer level are measured using ELISA

Example 35. This example illustrates various composite formulations of a calcium phosphate adjuvant.

Calcium phosphate is prepared according to example 17. The selected active agent is prepared to a concentration at 0.5 mg/ml in hydration media such as saline and in some instances adsorbed with the supplemental material. The calcium phosphate is hydrated using the media containing the active agent. Supplemental materials are added to the hydrated calcium phosphate by mere mixing at a concentration of 0.5 mg/ml. 0.5 ml of each adjuvant paste is injected subcutaneously into mice. Table 3 represents the various formulations of the inventive adjuvant. Each formulation is evaluated according to example 18. Additionally, in those formulations involving an antigen or antigens, blood samples are taken and sera are isolated. The respective antibody titers are measured using ELISA.

What is claimed is:

11. A composition of claim 1 further comprising a cytokine.

12. A composition of claim 11, wherein said cytokine is selected from: IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13, G-CSF, IL-15, GM-CSF, OSM, LIF, IFN-γ, IFN-α, IFN-β, B7.1, B7.2, TNF-α, TNF-β, LT-β, CD40 ligand, Fas ligand, CD27 ligand, CD30 ligand, 4-1BBL, IL-8, MCP-1, MIP-α, MIP-β, RANTES, TGF-β, IL-1α, IL-1β, IL-1 RA, IL-10, IL-12, and MIF.

13. A method for stimulating an immune response in a mammal, said method comprising administering to the mammal a composition comprising amorphous calcium phosphate.

14. A method for increasing immunogenicity of an antigen in a mammal, said method comprising co-administering both the antigen a composition comprising amorphous calcium phosphate.

15. An adjuvant composition comprising a first adjuvant, where said first adjuvant comprises poorly crystalline apatitic calcium phosphate.

16. A composition of claim 15, further comprising particles of said first adjuvant.

17. A composition of claim 16, wherein said particles have a diameter between 0.1 nm and 900 nm.

18. A composition of claim 15, wherein 25-100% by weight of said composition consists of said particles having a diameter between 0.1 nm and 900 nm.

19. A composition of claim 15, wherein said first adjuvant is strongly resorbable.

29. A composition of claim 28, further comprising particles of said first adjuvant.

30. A composition of claim 28, wherein said first adjuvant is strongly resorbable.

31. A composition of claim 28, formulated as an injectable paste.

32. A composition of claim 28, wherein said adjuvanticity enhancing means is a second adjuvant.

33. A composition of claim 32, wherein said second adjuvant is selected from: muramyl dipeptide, aluminum hydroxide, aluminum phosphate, hydroxyapatite, Incomplete Freund's Adjuvant, Complete Freund's Adjuvant and polymers.

34. A composition of claim 28 further comprising an antigen.

35. A composition of claim 28, wherein said adjuvanticity enhancing means is a cytokine.

36. A composition of claim 35, wherein said cytokine is selected from: IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13, G-CSF, IL-15, GM-CSF, OSM, LIF, IFN-γ, IFN-α, IFN-β, B7.1, B7.2, TNF-α, TNF-β, LT-β, CD40 ligand, Fas ligand, CD27 ligand, CD30 ligand, 4-1BBL, IL-8, MCP-1, MIP-α MIP-β, RANTES, TGF-β, IL-1α, IL-1β, IL-1 RA, IL-10, IL-12, and MIF.

37. A method for stimulating an immune response in a mammal, said method comprising administering to the mammal a composition comprising calcium phosphate and an adjuvanticity enhancing means.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/21182

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :424/278.1, 602, 484, 489: 514/769
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/278.1, 602, 484, 489: 514/769

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

West, CAPLUS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2,967,802 A (TOWEY et al) 10 January 1961, col. 1, lines 53-71, col. 2, lines 1-20, col. 3, lines 24-56.	1-2, 6-7, 10, 13-14, 28-31, 34
X	US 4,016,252 A (RELYVELD) 05 April 1977, see abstract, cols. 11-12.	1-2, 6-7, 13-14, 28-31, 34, 37
X	GUPTA et al. Adjuvant Properties of Aluminum and Calcium Compounds. Vaccine Design. Chapter 8, 1995,pages 229-248, especially pages 240-241.	1-2, 6, 10-11, 13-16, 19, 23-24, 26-30, 34-35, 37
Y	US 4,110,432 A (WILKINSON et al) 29 August 1978, cols. 1-2, col. 8, lines 43-57, col. 9, lines 1-35.	1-37

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

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08 DECEMBER 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/21182

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,462,751 A (KOSSOVSKY et al) 31 October 1995, cols. 3-4.	1-37
Y	RELYVELD et al. Calcium Phosphate Adjuvanted Allergens. Annals of Allergy. June 1985, Vol. 54, pages 521-529, entire document.	1-37
A	US 5,676,976 A (LEE et al) 14 October 1997, cols. 6-8.	1-7, 15-20, 28-31.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/21182

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 09/14, 45/00, 47/00; A01N 25/00

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